REMARKS

Reconsideration of this application is respectfully requested.

Claims 133-141 have been canceled. New claims 142-149 are derived from canceled claims 133-141 and are fully supported by the specification, for example, on page 13, lines 6-15, where the claimed HIV-1 LTR fragment is disclosed. Upon amendment, claims 142-149 are pending in this application. No new matter enters by amendment.

Claims 133-141 were rejected under 35 U.S.C. § 102(e) as allegedly being anticipated by U.S. Patent No. 6,001,977 ("the Chang patent"). The Examiner contends that the Chang patent discloses a complete 3' LTR. Applicants traverse the rejection.

Applicants claim priority of Appln. GB 84 29099, filed on November 16, 1984, under 35 U.S.C. § 119. A certified copy of Appln. GB 84 29099 was filed in this application on October 21, 1993. The Office has acknowledged applicants' claim for priority of Appln. GB 84 29099 under 35 U.S.C. § 119. (*See* Paper No. 8.) Therefore, in order to be effective prior art, the Chang patent must have disclosed applicants' claimed invention prior to November 16, 1984. The Chang patent does not make such a disclosure.

The Chang patent is a division of application No. 06/693,866 ("the '866 application"), filed January 23, 1985, which is a continuation-in-part of application No. 06/659,339 ("the '339 application"), filed October 10, 1984, which is a continuation-in-part of application No. 06/643,306 ("the '306 application"), filed August 22, 1984. Since the filing date of the '866 application is ***after*** November 16, 1984, the question becomes

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whether the disclosures in the '339 application and '306 application anticipate applicants' claims.

Applicants' claims 142-149 recite an LTR fragment. This fragment is located in the R region of the HIV-1 LTR. Neither the '339 application nor the '306 application discloses clones containing the fragment.

The '306 application (Exhibit 1) discloses 3 HIV-1 clones: BH5, BH8, and BH10. ('306 application at 4, last paragraph.) As discussed in detail below, these clones do not contain the fragment because they are missing a part of the HIV-1 LTR. Likewise, the '339 application (Exhibit 2) concedes that an approximately 200 bp fragment of the HIV-1 LTR is missing from the HIV-1 clones. ('339 application at 9, first paragraph, and at Fig. 1.)

The Chang patent explains that 182 base pairs of HIV-1 DNA "including a portion of R, U5, the tRNA primer binding site and a portion of the leader sequence" were missing from clone BH10. U.S. Patent No. 6,001,977 at 8, col. 2, lines 46-50. An analysis of Figs. 3a and 3i of the Chang patent reveals the precise location of the fragment of the HIV-1 LTR missing in Chang's BH5, BH8, and BH10 clones.

Fig. 3a of the Chang patent shows that clones BH5, BH8, and BH10 are missing a fragment from nucleotide position 40 to nucleotide position 222. At both of the termini of the missing fragment is the sequence "GAGCTC," which specifies an *Sst*I site. (*See* yellow highlights on copy of Fig. 3a attached as Exhibit 3.) Fig. 3a further indicates that the missing region encompasses part of the "R" region of the LTR, all of the "U5" region of the LTR, the "tRNA-lysine" region, and part of the "Leader sequence" region.

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The 3' LTR region of Chang's clones does not contain any of these missing sequences. Specifically, Fig 3i of the Chang patent shows that clones BH5, BH8, and BH10 are missing a fragment from nucleotide position 9154 to the end of the HIV-1 LTR region. At the termini of clones BH8 and BH10 is the sequence "GAGCTC," which specifies an *Sst*I site. (*See* yellow highlight on copy of Fig. 3i attached as Exhibit 4.) Fig. 3i further indicates that the missing region encompasses part of the "R" region of the LTR, and all of the "U5" region of the LTR.

Thus, it is evident that Chang's BH5, BH8, and BH10 clones were missing part of the "R" region of the LTR, all of the "U5" region of the LTR, the "tRNA-lysine" region, and part of the "Leader sequence" region. The missing DNA sequences are not present in either the 5' or 3' LTR regions of Chang's clones. Consequently, Chang's clones were incomplete. Since Chang's clones did not contain these regions, Chang's clones could not contain a complete HIV-1 LTR.

Applicants' new claims 142-149 recite an HIV-1 LTR fragment, which is missing from Chang's clones. The attached copies of Fig. 3a and 3i of the Chang patent (Exhibits 3 and 4) show the location of the fragment in blue highlighting. The fragment localizes to the part of the "R" region that is missing in Chang's BH5, BH8, and BH10 clones. Consequently, Chang's clones disclosed in the '306 and '339 applications did not contain the fragment and cannot anticipate applicants' claims. Accordingly, applicants respectfully request withdrawal of the rejection.

Claims 133-141 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter that was not described in the specification in such a way as to convey to the skilled artisan that applicants had possession of the claimed

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invention at the time that the application was filed. The Examiner contends that applicants' claims do not specify any specific structure.

As discussed above, applicants' new claims 142-149 recite an HIV-1 LTR fragment. This fragment provides a common attribute possessed by the members of the claimed genus. Accordingly, applicants respectfully submit that the rejection is inapplicable to new claims 142-149.

Applicants respectfully submit that this application is now in condition for allowance. In the event that the Examiner disagrees, he is invited to call the undersigned to discuss any outstanding issues remaining in this application in order to expedite prosecution.

Please grant any extensions of time required to enter this response and charge any additional required fees to our deposit account 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.

Dated: August 1, 2003

By: _____

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SN 643,306
filed 8/22/84

APPLICATION FOR UNITED STATES PATENT

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Title: MOLECULAR CLONES OF THE GENOME OF HTLV-III

Abstract of the Disclosure

Disclosed is the molecular cloning of HTLV-III, the adult leukemia and acquired immune deficiency syndrome (AIDS) virus. Clone BH10 contains a 9.0 Kb viral insert constituting the entire HTLV-III genome. Clones BH8 and BH5 contain viral inserts of 5.5 Kb and 3.5 Kb, respectively. These clones are suitable for the development of diagnostic and therapeutic measures for AIDS, as well as use as probes for the detection of AIDS.

In related inventions, HTLV-III was detected, isolated, and immortalized in an HT cell line. Since evidence now strongly indicates that HTLV-III is related to acquired immune deficiency syndrome (AIDS), the ability to enhance production of the virus and determine the DNA sequences of the virus is critically important to developing a cure or reagent active against AIDS. The present invention takes one such significant step by disclosing the process for molecularly cloning the complete genome of the HTLV-III virus. In short, The molecular cloning of the complete genome of the HTLV-III virus produced by one of these lines designated H9/HTLV-III is disclosed. Two forms of this virus are identified which are highly related but differ in several restriction enzyme cleavage sites. Both variants exist as integrated and unintegrated forms in the infected cell line. The complete genomes of two forms of HTLV-III are molecularly cloned and shown to exist in the long-term infected cell line both as polyclonally integrated provirus and as unintegrated viral DNA. These clones are used as probes to detect viral sequences in cell lines other than H9/HTLV-III, taken from different AIDS patients, and in fresh lymphoid tissues of AIDS patients, providing further evidence that the cloned genomes constitute predominant forms of HTLV-III, the causative agent in AIDS.

Statement of Utility

Previous work with the HTLV family of virus showed three variants. Of these, it was believed that HTLV-III was the causative agent of AIDS. Using the clones produced by this invention, HTLV-III has been shown to be distinctly different than HTLV-I and HTLV-II, whereas HTLV-I and -II share greater homology and thus better identification of AIDS virus in sera.

Description of the Figures

Figure 1 is a Southern blot analysis of unintegrated DNA of HTLV-III. No viral sequences could be detected in the undigested DNA after 4 hours. However, a major species of viral DNA of approximately 10 Kb length was present in the 10, 15, 24 and 48 hr harvest representing the linear unintegrated form of the virus. A representative Southern blot of the 15 hr harvest digested with several restriction enzymes is shown in this figure. Methods: 8 x 10^8 fresh uninfected H9 cells were infected with concentrated supernatant from cell line H9/HTLV-III containing 4 X 10^{11} particles of HTLV-III. Infected cells were divided into five Roller bottles and harvested after 4, 10, 15, 24 and 48 hrs. Low molecular weight DNA was prepared using the Hirt fractionation procedure and 30 ug of undigested and digested DNA were separated on a 0.8% agarose gel, transferred to nitrocellulose paper and hybridized to a HTLV-III cDNA probe for 24 hr at 37°C in 1 X SSC, 40% formamide and 10% Dextran sulfate. cDNA was synthesized from poly(A) selected RNA prepared from doubly banded HTLV-III virus in the presence of oligo(dT) primers. Filters were washed at 1 X SSC at 65°C.

Figure 2 is a restriction endonuclease map of two closely related HTLV-III variants cloned from unintegrated viral DNA. Three recombinant clones (λ BH10, λBH5 and λBH8) were analyzed and their inserts (9 Kb, 5.5 Kb and 3.5 Kb, respectively) were mapped with the indicated enzymes. They represent two variant forms of HTLV-III differing in three enzyme sites which are depicted in bold letters and by an asterisk. As SstI cuts the LTR of the HTLV-III the three clones represent two full length genomes with one LTR. A schematic map of this viral genome is shown at the bottom of the

figure, although the total length of the LTR is approximate. Methods: Low molecular weight DNA combined from the 15 and 24 hr harvest was fractionated on a 10-40% sucrose gradient. Aliquots of the fractions were electrophoresed on a 0.5% agarose gel, transferred to nitrocellulose paper and hybridized to HTLV cDNA under conditions described in Figure 1. Fractions which contained the unintegrated linear HTLV-III genome shown by hybridization were pooled, the DNA was subsequently digested with SstI and ligated to phosphatase treated SstI arms of λgtWes·λB. After in vitro packaging, recombinant phages were screened for viral sequences with HTLV-III cDNA.

Figure 3 demonstrates HTLV-III viral sequences in the infected cell line H9/HTLV-III. Both variant forms of HTLV-III were detected as integrated provirus as well as unintegrated viral DNA in the infected cell line. However, no viral sequences were found in uninfected H9 cells, uninfected HT cells nor in normal human thymus (NT). Methods: 10 µg of high molecular weight DNA were digested with restriction enzymes as indicated and hybridized to nick translated phage insert from BH10 under the same conditions as described in Figure 1.

Figure 4 shows a sequence homology of HTLV-III to other members of the HTLV family. A schematic restriction map of HTLV-I, HTLV-Ib and HTLV-II is drawn below indicating the length and the location of the generated fragments in respect to the corresponding genomic regions. LTR, gag, pol, env and pX regions are drawn to scale according to the published nucleotide sequence of HTLV-I. The bands which are most highly conserved as stringency increases correspond to the gag/pol junction region of HTLV-I (1.8 Kb PstI fragment) and HTLV-IIb (3.1 Kb PstI fragment) and to the 3' part

of the pol region of HTLV-II (2.1 Kb SmaI/BamHI fragment) which do not overlap assuming the same genomic organization in HTLV-II. Fragments corresponding to pX of HTLV-I (2.1 Kb SstI Pst fragment) and HTLV-Ib (1.4 Kb Pst fragment) are less conserved but still visible at Tm - 28°C on the original autoradiogram. Digestion of GaLV generates six fragments, none of which show hybridization under medium or high stringency. Methods: Subclones of full length genomes of a prototype HTLV-I, HTLV-Ib, HTLV-III and GaLV (Seato strain) were digested with the following enzymes, PstI plus SstI (HTLV-I and HTLV-Ib), BamHI plus SmaI (HTLV-II) and Hind III plus SmaI plus XhoI (GaLV). Four replicate filters were prepared and hybridized for 36 hr under low stringency (8 X SSC, 20% formamide, 10% Dextran sulfate at 37°C) to nick translated insert of λ BH10. Filters were then washed in 1 X SSC at different temperatures, 22°C (Tm - 70°C) filter 1, 37°C (Tm - 56°C) filter 2, 50°C (Tm - 42°C) filter 3 and 65°C (Tm - 28°C).

The Invention

The present invention discloses a method for production of molecular clones of HTLV-III from a fraction enriched for the unintegrated provirus in acutely infected cells. Three clones for the HTLV-III genome were produced using recombinant DNA techniques by isolating and characterizing unintegrated viral DNA, cleaving this DNA with the appropriate restriction enzyme, and constructing a phage library capable of being screened by viral cDNA. This process led to the production of three clones: BH10, containing a viral insert of 9.0 Kb corresponding to the complete HTLV-III genome; clone BH8 containing an insert of 5.5 Kb; and clone BH5 containing a viral insert of 3.5 Kb. See Figure 3 for a pictoral representation of the differences between these three clones.

In general, cloning the HTLV-III genome involved isolating unintegrated viral DNA after infection of H9-cells with concentrated HTLV-III virus and cloning this DNA in a lambda phage library to be screened with viral cDNA. The cell line H9/HTLV-III produces large quantities of HTLV-III virus and serves as the principal producer cell line for immunological assays used to detect virus specific antigens and antibodies in AIDS sera. Cultures of H9/HTLV-III cells (infected cells) are grown and harvested, followed by extraction of low molecular weight DNA from the newly infected cells. This produces unintegrated viral DNA. A cDNA library is formed using HTLV-III cDNA. This cDNA is then used as a probe for assaying the unintegrated viral DNA. Unintegrated linear DNA (provirus DNA) is then obtained, containing the entire HTLV-III genome, i.e., replication competent. This DNA is then digested in plasmid lambda gt Wes · lambda B to form clone lambda BH10. The other clones are produced by digesting provirus DNA that does not contain the entire HTLV-III genome.

Two elements of the above process are recombinant DNA procedures, such as, the DNA library and a cDNA probe. The library is formed by taking the total DNA from H9/HTLV-III cells, cutting the DNA into fragments with a suitable restriction enzyme, hybridizing to the fragments to a radiolabeled cDNA probe, joining the fragments to plasmid vectors, and then introducing the recombinant DNA into a suitable host.

The cDNA probe is an HTLV-III cDNA probe made from double-banded HTLV-III mRNA. A short oligo-dT chain is hybridized to the poly-A tail of the mRNA strand. The oligo-T segment serves as a primer for the action of reverse transcriptase, which uses the mRNA as a template for the synthesis of a complementary DNA strand. The resulting cDNA ends in a hairpin loop.

Once the mRNA strand is degraded by treatment with NaOH, the hairpin loop becomes a primer for DNA polymerase I, which completes the paired DNA strand. The loop is then cleaved by S1 nuclease to produce a double-stranded cDNA molecule. Linkers are then added to the double-stranded cDNA by using DNA ligase. After the linkers are cut open with a restriction enzyme and the cDNA is inserted into a suitable plasmid cleaved with the same enzyme, such as pBR322. The result is a cDNA-containing recombinant plasmid.

Statement of Deposit

The cell lines and clones of this invention are on deposit in the American Type Culture Collection in the manner prescribed by the Patent and Trademark Office with regard to permanence of the deposit for the life of the patent and without restriction on public access. The accession numbers are: H9/HTLV-III, CRL 8543; BH10, #40125; BH8, #40127; and BH5, #40126.

Specific Disclosure

Concentrated virus from H9/HTLV-III is used to infect fresh uninfected H9 cells at a multiplicity of 50 viral particles/cell; cultures are harvested after 4, 10, 15, 24 and 48 hours. Extrachromosomal DNA is extracted according to the procedure of Hirt and assayed for its content of unintegrated viral DNA using HTLV-III cDNA as a probe. This cDNA is primed by oligo(dT) and copied from poly(A) containing RNA from virions that had been twice banded on sucrose density gradients. Unintegrated linear viral DNA is first detected after 10 hrs and is also present at the subsequent time points. A Southern blot of the 15 hr harvest is shown in Figure 1. A band of approximately 10 Kb in the undigested DNA represents the linear form of the unintegrated,

replication-competent HTLV-III. No closed or nicked circular DNA could be detected in the 10, 15 and 24 hour harvest, but both forms were evident in small amounts at the 48 hr harvest (data not shown). The viral genome was not cut by XbaI, whereas SstI generated three predominant bands of 9 Kb, 5.5 Kb and 3.5 Kb (Figure 1). These bands represent the complete genomes of two forms of HTLV-III, both cut by SstI in the LTR and one having an additional SstI site in the middle of its genome. Clone BH10 contains a viral insert of 9.0 Kb, a size consistent with the complete HTLV-III genome. Clones BH8 and BH5 contain inserts of 5.5 Kb and 3.5 Kb, respectively, and together they overlap completely with BH10, except for a few restriction enzyme sites polymorphisms in BH5. Therefore, BH10 and BH8 plus BH5 represent two variants of HTLV-III.

EXAMPLE 1

In order to demonstrate the presence of these two variants in the original cell line, nick-translated inserts of lambda BH10 was hybridized to a Southern blot of H9/HTLV-III genomic cDNA digested with several restriction enzymes (Figure 3). Both forms could be detected using the enzyme SstI generating the expected 3 bands of 9.0 Kb, 5.5 Kb and 3.5 Kb XbaI which does not cut the provirus generating a high molecular weight genome representing polyclonal integration of the provirus and a band of approximately 10 Kb which could be interpreted as representing unintegrated viral DNA since a band of identical size was also present in the undigested first preparation (Figure 1). This was confirmed by Southern blot hybridization of undigested cellular DNA. The existence of unintegrated viral DNA thus explains the presence of a 4 Kb and 4.5 Kb EcoRI fragment seen in both first and total cellular DNA preparations (Figure 1 and Figure 3). BglII and HindIII

both cut the LTR and generated the expected internal bands. Several faint bands in the HindIII digest, in addition to the internal bands, represent either defective proviruses or another variant form with differences in the HindIII restriction pattern. The lack of HTLV-III sequences in the uninfected H9 cell line and the uninfected parental line HT as well as in normal human thymus demonstrated the exogenous nature of HTLV-III and showed that the virus does not contain any human cellular sequences. The same results were obtained using nick-translated inserts from lambda BH5 and lambda BH8.

EXAMPLE 2

The availability of the cloned HTLV-III genome allowed sequence homology between HTLV-III, HTLV-I and HTLV-II to be evaluated. Replicate Southern blots of restriction enzyme digested clones representing the complete genomes of HTLV-I, HTLV-Ib, HTLV-II and GALV as a control were hybridized to full length HTLV-III probe under relaxed conditions. The filters were then washed (λ BH10i) under conditions of low, medium, and high stringencies in order to estimate the extent of homology between HTLV-III and these viruses (Figure 4). This experiment showed that there is specific homology between HTLV-III, HTLV-I, HTLV-Ib and HTLV-II but not with HTLV-III and GALV. As demonstrated, hybridization of HTLV-III to other members of the HTLV family could be detected at the values of -42°C and -28°C, conditions under which no hybridization to GALV was seen (Figure 4, panels C and D). Of note, the restriction fragments showing greatest homology correspond to the gag/pol region of HTLV-I and to an apparently non-overlapping portion of the pol region of HTLV-II (assuming that the genomic arrangement is similar to that of HTLV-I). Further analysis revealed that it is the 5' half of the

gag and the gap between gag and pol which has the greatest homology in HTLV-I. Finally, in HTLV-Ib (a variant of HTLV-I) hybridization to the px region could be seen (1.4 Kb Pst fragment) as well as to the corresponding px fragment in HTLV-I (2.1 Kb Pst/Sst) on the original autoradiogram.

EXAMPLE 3

Figure 2 shows the restriction map of three clones designated λBH10, λBH5 and λBH8 which correspond in size to the three SstI fragments shown in Figure 1. Comparison of these maps suggests that λBH5 plus λBH8 constitute one HTLV-III genome, and λBH10 another. The two viral forms differ in only three out of 21 mapped enzyme sites, including the internal SstI site. As expected, the phage inserts of λBH5 and λBH8 hybridize under high stringency conditions to λBH10 but not to each other as analyzed by Southern blot hybridization and electron microscopic heteroduplex analysis. To show the presence of LTR sequences in the clones and to determine their orientation, a cDNA clone (C15) was used as a probe and contained U3 and R sequences. This clone strongly hybridized to the 0.5 Kb BglII fragment of λBH10 and λBH8, orienting this side 3', and faintly hybridized to the 0.7 Kb SstI/PstI fragment of λBH5 and λBH10, orienting this side 5', and demonstrated that SstI cuts the LTR of HTLV-III in the R region.

EXAMPLE 4

The presence of two variant forms of HTLV-III in the original cell line was demonstrated by hybridizing the radiolabelled insert of λBH10 to a Southern blot of H9/HTLV-III genomic DNA digested with several restriction enzymes (Figure 3). Both forms were detected using the enzyme SstI which generated the

expected 3 bands of 9Kb, 5.5 Kb and 3.5 Kb length. Both of these forms are also present as integrated proviruses because they have been cloned along with their flanking cellular sequences from a genomic library of H9/HTLV-III. Furthermore, XbaI, which does not cut the provirus, generated a high molecular weight smear representing polyclonal integration of the provirus and a band of approximately 10 Kb, representing unintegrated viral DNA. This same 10 Kb band was also detected in undigested H9/HTLV-III DNA, again indicating unintegrated viral DNA. The presence of unintegrated viral DNA also explains the 4 Kb and 4.5 Kb EcoRI fragment seen in both the Hirt and total cellular DNA preparations (Figures 1, 3). Bgl II and Hind III both cut the LTR and generate the expected internal bands. Several faint bands, in addition to the internal bands using Hind III, represent either defective proviruses or another variant form present in low copy number. The lack of HTLV-III sequences in the DNA of the uninfected H9 cell line and the uninfected parental cell line HT as well as in normal human thymus clearly demonstrates the exogenous nature of HTLV-III and shows that the virus does not contain human cellular sequences. The same results were obtained using λBH5 and λBH8 as probe inserts.

WE CLAIM

1. Recombinant clone BH10 characterized by containing the complete HTLV-III genome.

2. Recombinant clone BH8 characterized by containing a 5.5 Kb viral insert from HTLV-III virus.

3. Recombinant clone BH5 characterized by containing a 3.5 Kb viral insert from HTLV-III.

4. A process for the production of recombinant molecular clones of HTLV-III consisting essentially of cleaving unintegrated viral DNA from HTLV-III cells with a restriction enzyme to obtain a provirus, hybridizing radiolabeled cDNA to said provirus, and digesting said virus in a suitable plasmid.

5. A process for the molecular cloning and expression of a cDNA sequence of HTLV-III consisting essentially of

isolating total cellular mRNA from H9/HTLV-III cells;

forming double-stranded cDNA from said mRNA and inserting said double-stranded cDNA into a phage lambda to form a recombinant DNA molecule;

hybridizing said recombinant DNA molecule with a radiolabelled probe;

removing cDNA from said molecules and inserting said cDNA into a suitable plasmid; and

transfecting said plasmids into a suitable host cell capable of expressing HTLV-III DNA sequences.

6. A process of Claim 5 wherein said plasmid is λBH10.

7. A process of Claim 5 wherein said plasmid is λ BH8.

8. A process of Claim 5 wherein said plasmid is λ BH5.

9. A process of Claim 5 wherein said cDNA sequence corresponds to a 9.0 Kb sequence.

10. A process of Claim 5 wherein said cDNA sequence corresponds to a 5.5 Kb sequence.

11. A process of Claim 5 wherein said cDNA sequence corresponds to a 3.5 Kb sequence.

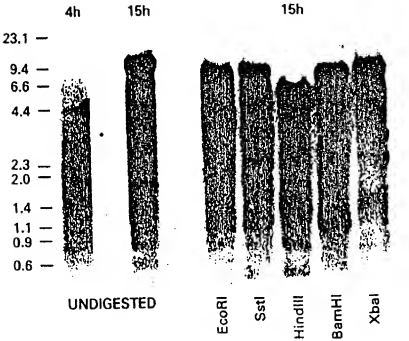
4h
15h
15h
23.1
9.4
6.6
4.4
2.3
2.0
1.4
1.1
0.9
0.6
UNDIGESTED
EcoRI
SstI
HindIII
BamHI
XbaI

Fig 1

HTLV-III a
LTR
Sst I
Hind III
Pst I
Hind III
Bgl II
Kpn I
Kpn I
EcoR I
EcoR I
Sal I
Kpn I
Bgl II
Hind III
BamH I
Xho I
Kpn I
Bgl II
Bgl II
Sst I
LTR
HBH - 10
HTLV-III b
LTR
Sst I
Hind III
Pst I
Hind III
Bgl II
Kpn I
Kpn I
EcoR I
EcoR I
Sal I
*Sst I
LTR
Sst I *
Kpn I
Bgl II
Hind III
BamH I
Hoa I *
Xho I
Kpn I
Bgl II
BamH I *
Bgl II
Sst I
LTR
HBH - 5
HBH - 8
5'
3'
LTR
Pol
LTR
0
1
2
3
4
5
6
7
8
kb

FIG 2

Fig #4 DWS 8/17/84

8/17/84

8/17/84

MPJ 8/17/84

INFECTED
H9/HTLV-III
UNINFECTED
H9 HT NT
23.1
9.4
6.6
4.4
2.3
2.0
1.4
1.1
0.9
0.6
XbaI
SstI
BglII
HindIII
EcoRI
SstI

FIG 3

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

MOLECULAR CLONES OF THE GENOME OF HTLV-III

the specification of which

(check one)

☒ is attached hereto.

☐ was filed on _____ as

Application Serial No. _____

and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

None

Form PTO-FB-110 (8-83) Patent and Trademark Office-U.S. DEPARTMENT OF COMMERCE

Prior Foreign Application(s) — Priority Claimed

(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
None				
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
None		
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

English Language Declaration

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (*list name and registration number*)

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FILING DATE:	
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ASSIGNMENT (JOINT)
(Executive Order)

Mikulas
HAHN & Popovic

WHEREAS, we, FLOSSIE WONG-STAAL, ROBERT C. GALLO and BEATRICE H. employees of the U.S. Public Health Service, Department of Health and Human Services, and citizens of the United States, have invented

MOLECULAR CLONES OF THE GENOME OF HTLV-III

for which we are about to make application, executed Aug. 17 1984 to the Commissioner of Patents for grant of Letters Patent of the United States; and

WHEREAS, we are the applicants named in the above identified application for Letters Patent; and

WHEREAS, the conditions under which said invention was made are such as to entitle the Government under Paragraph 1(a) of Executive Order 10096, to the entire right, title and interest therein, including foreign rights; and

WHEREAS, as to foreign rights, it is the policy of the Government to obtain an option to exercise such rights;

NOW, THEREFORE, to all whom it may concern; be it known that for and in consideration of the premises and other valuable considerations, we the undersigned, have sold, assigned and transferred and by these presents do sell, assign and transfer unto the Government of the United States of America as represented by the Secretary of the Department of Health and Human Services, the entire right, title and interest throughout the United States of America, its territories and dependencies, in and to the aforesaid invention described in the aforesaid application for Letters Patent of the United States, and all Letters Patent issuing thereon and any continuations, divisions and reissues or extensions thereof; hereby authorize and request the Commissioner of Patents to issue said Letters Patent to the Government of the United States of America, as represented by the Secretary of the Department of Health and Human Services, and his successors, as assignee of the entire right, title and interest in and to the same throughout the United States of America, its territories and dependencies, for the sole use for the full term or terms for which said Letters Patent and any continuations, divisions and reissues or extensions thereof are, or may be, granted as fully and entirely as the same would have been held by us, had this assignment not been made, and we do hereby grant unto the Government of the United States as represented by the Secretary of the Department of Health and Human Services, the option to take all of the right, title and interest in said invention or all applications for Letters Patent thereon in all countries foreign to the United States in which the Government of the United States may file, or cause to be filed, applications for Letters Patent, without payment to me of any further consideration; provided, however, that this grant of an option to take foreign rights in my invention, or applications for Letters Patent thereon, shall have force and effect only as to such applications filed in foreign countries within six months of the filing date of any applications for United States Letters Patent covering my invention, and that all foreign rights not exercised under the option are left to us such [illegible].

INVENTOR:

Flossie Wong-Staal
FLOSSIE WONG-STAAL

SUBSCRIBED AND SWORN to before me this 17th day of August 19 84; at Bethesda in the County of Mo and State of Md

Rosanne A. Hyman
NOTARY

(SEAL)

My Commission Expires on July 1, 1986

INVENTOR:

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ROBERT C. GALLO

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INVENTOR:

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(SEAL)

My Commission Expires on July 1, 1986

My Commission Expires on July 1, 1986

Subscribed and sworn to before me, in my presence, this 17th day of August 1984 a Notary Public in and for the State of Maryland

Rosanne A. Hyman
Notary Public

My commission expires _____ 19

My Commission Expires on July 1, 1986

CLONING AND EXPRESSION OF HTLV-III DNA

Description

Technical Fields

This invention is in the fields of biology and virology and in particular relates to human T cell leukemia virus - type III (HTLV-III).

Background Art

The term human T cell leukemia-lymphoma virus (HTLV) refers to a unique family of T cell tropic retroviruses. Such viruses play an important role in the pathogenesis of certain T cell neoplasms. There are presently three known types of HTLVs. One subgroup of the family, HTLV-type I (HTLV-I) is linked to the cause of adult T-cell leukemia-lymphoma (ATLL) that occurs in certain regions of Japan, the Caribbean and Africa. HTLV-type II (HTLV-II) has been isolated from a patient with a T-cell variant of hairy cell leukemia. M. Popovic et al., Detection, Isolation, and Continuous Production of Cytopathic Retroviruses (HTLV-III) from Patients with AIDS and Pre-AIDS, Science 224:497-

HTLV-type III (HTLV-III) has been isolated from many patients with acquired immune deficiency syndrome (AIDS). It refers to prototype virus isolated from AIDS patients. Groups reported to be at greatest risk for AIDS include homosexual or bisexual males; intravenous drug users and Haitian immigrants to the United States. Homosexual or bisexual males having heterosexual contacts,

hemophiliacs who receive blood products pooled from donors and recipients of multiple blood transfusions are also at risk. Clinical manifestations include severe, unexplained immune deficiency which generally involves a depletion of helper T lymphocytes. These may be accompanied by malignancies and infections. The mortality rate for those with AIDS is high. A less severe form of AIDS also exists, in which there may be lymphadenopathy and depressed helper T cell counts; there is not, however, the devasting illness characteristic of full-blown AIDS. There are many individuals, who are classified as having early AIDS (pre-AIDS), who exhibit these signs. It is not now possible to predict who among them will develop the more serious symptoms.

Much of the evidence implicates HTLV-III as the etiological agent of the infectious AIDS. First, there is consistent epidemiology; greater than 95% of the patients with AIDS have antibodies specific for HTLV-III. Second, there has been reproducible identification and isolation of virus in this disease; more than 100 variants of HTLV-III have been isolated from AIDS patients. Third, there has been transmission of the disease to normal healthy individuals who received blood transfusions from infected blood donors.

HTLV-III has been shown to share several properties with HTLV-I and HTLV-II but also to be morphologically, biologically and antigenically distinguishable. R.C. Gallo et al., Frequent Detection and Isolation of Cytopathic Retroviruses (HTLV-III) from Patients with AIDS and At Risk for AIDS. Science, 224:500-503. (1984). For example,

HTLV-III has been shown to be antigenically related to HTLV-I and HTLV-II by demonstrating cross-reactivity with antibodies to HTLV-I and HTLV-II core proteins, P24 and P19, and envelope antigens and by nucleic acid cross-hybridization studies with cloned HTLV-I and HTLV-II DNAs. However, unlike HTLV-I and HTLV-II, it lacked the ability to infect and transform T cells from normal umbilical cord blood and bone marrow in vitro, and has the cytopathic effect on infected cells only.

Like the RNA genome of other retroviruses, the RNA genome of HTLV-III contains three genes which encode viral proteins: 1) the gag gene, which encodes the internal structural (nucleocapsid or core) proteins; 2) the pol gene, which encodes the RNA-directed DNA polymerase (reverse transcriptase); and 3) the env gene, which encodes the envelope glycoproteins of the virion. In addition, the HTLV-III genome contains a region designated Px, located between the env gene and the 3' LTR, which appears to be involved in functional killing of the virus.

At this time, AIDS is still difficult to diagnose before the onset of clinical manifestations and impossible to treat or even prevent.

Summary of the Invention

This invention is based upon applicant's cloning of HTLV-III DNA in recombinant/vector host systems capable of expressing immunoreactive HTLV-III polypeptides. In one embodiment, an immuno-reactive protein coded for by an env gene sequence of HTLV-III has been produced by these recombinant

DNA methods. This polypeptide is immunoreactive with sera of patients having acquired immunodeficiency syndrome or antibodies to HTLV-III. The polypeptide expressed has been isolated.

In another embodiment of the invention, immunoreactive polypeptides produced by the recombinant DNA methods are employed in the production of antibodies, including monoclonal antibodies, reactive with the polypeptides. Such antibodies form the basis for immunoassay and diagnostice techniques for detecting HTLV-III, particularly in body fluids such as blood, saliva, urine, etc.

In another embodiment of the invention, DNA probes are formed from DNA sequences coding for portions of the HTLV-III genome. Such DNA probes can also be employed in detecting the presence of HTLV-III in blood or other fluids.

Diagnostic kits including immunoreactive polypeptides, DNA probes, etc. can also be produced to include any of the products of this invention.

Brief Description of the Figures

Figure 1 is a representation of HTLV-III DNA. Figure 1a shows sites at which the genome is cut by the restriction enzyme SstI and Figure 1b shows the fragments of HTLV-III genome produced through the action of restriction enzymes Kpn, EcoRI and Hind III.

Figure 2 is a representation of HTLV-III DNA and the location of restriction enzyme sites in the genome.

Figure 3 shows nucleotide sequences for HTLV-III DNA which encompasses the env region.

Figure 4 is an immunoblot showing the position on an SDS polyacrylamide gel of HTLV-III env-Beta-galactosidase fusion proteins.

Best Mode of Carrying Out the Invention

The envelope glycoprotein is the major antigen recognized by the antiserum of AIDS patients. In this respect, HTLV resembles other retroviruses, for which the envelope glycoprotein is typically the most antigenic viral polypeptide. In addition, the neutralizing antibodies are generally directed toward the envelope glycoprotein of the retrovirus. Serum samples from 88 percent to 100 percent of those with AIDS have been shown to have antibodies reactive with antigens of HTLV-III; the major immune reactivity was directed against p41, the presumed envelope antigen of HTLV-III. Antibodies to core proteins have also been demonstrated in serum of AIDS patients, but are evidently not as effective an indicator of infection as is the presence of antibodies to envelope antigen.

The p41 antigen of HTLV-III has been difficult to characterize because the viral envelope is partially destroyed during the process of virus inactivation and purification. The present invention responds to the great need to characterize the antigenic component of the HTLV-III virus--and thus provide screening, diagnostic and preventive products and methods--in several ways.

First, the present invention relates to the isolation of genes of HTLV-III which encode

immunoreactive polypeptides; identification of the nucleotide sequence of these genes; introduction of DNA sequences specific to these viral DNA sequences into appropriate vectors to produce viral RNA and the formation of DNA probes. These probes are comprised of sequences specific to HTLV-III DNA and are useful, for example, for detecting the same HTLV-III DNA sequences in body fluids (e.g., blood).

Second, the present invention relates to HTLV-III polypeptides which are produced by translation of the recombinant DNA sequences encoding HTLV-III proteins. Polypeptides which are so produced and which are immunoreactive with serum from AIDS patients are referred to as recombinant DNA-produced immunoreactive HTLV-III polypeptides. They include, but are not limited to, antigenic HTLV-III core and envelope polypeptides which are produced by translation of the recombinant DNA sequences specific to the gag and the env DNA sequences encoding HTLV-III core proteins and envelope glycoproteins, respectively. They also include the polypeptides which are produced by translation of the recombinant DNA sequences specific to the Px genes of HTLV-III. The polypeptides may be used as vaccines for the prevention of AIDS. The methods of producing the polypeptides are also a subject of this invention, as are diagnostic methods based on these polypeptides.

Third, the present invention also relates to antibodies against the immunoreactive HTLV-III polypeptides which are the subject of this invention. These antibodies are the basis for assays

relating to the diagnosis of AIDS or the presence of HTLV-III in body fluids.

In one embodiment of this invention, genetic engineering methods are used to isolate DNA sequences of HTLV-III which encode immunoreactive HTLV-III polypeptides, such as the core protein and the envelope glycoprotein, and to identify the nucleotides which comprise those sequences. The proviral genes integrated into host cell DNA are molecularly cloned and the nucleotide sequences of the cloned provirus is determined.

An E. coli expression library of HTLV-III DNA is constructed; in this library are vectors harboring HTLV-III DNA sequences. The HTLV-III genome is cloned and cuts are then made in the cloned HTLV-III genome with restriction enzymes to produce DNA fragments. (Figures 1 and 2) HTLV-III DNA fragments of approximately 200-500bp are isolated from agarose gel, end repaired with T_4 polymerase and ligated to linker DNA. The linker ligated DNA is then treated with a restriction enzyme, purified from agarose gel and cloned in an expression vector. Examples of the expression vectors used are: OmpA, pIN (A, B and C), lambda pL, T7, lac Trp, ORF and lambda gt11. In addition, mammalian cell vectors such as pSV28pt, pSV2neo, pSVdhfr and VPV vectors, and yeast vectors, such as GAL1 and GAL10, may be used.

The bacterial vectors contain the lac coding sequences, into which HTLV-III DNA can be inserted for the generation of B-galactosidase fusion protein. The hybrid molecules are then introduced into bacteria (e.g., E.coli); those cells which take up a

vector containing HTLV-III DNA are said to be transformed. The bacteria are plated on top of MacConkey agar plates in order to verify the phenotype of clone. If functional B-galactosidase is being produced, the colony will appear red.

Bacterial colonies are also screened with HTLV-III DNA probes containing the DNA regions of interest (e.g., HTLV-III gag and env DNA sequences). This results in identification of those clones containing the insert. Clones which are positive when screened with the DNA probe and positive on the MacConkey agar plates are isolated.

This identification of cells harboring the HTLV-III DNA sequences makes it possible to produce HTLV-III polypeptides which are immunoreactive with HTLV-III specific antibody. The cells from the selected colonies are grown in culture under conditions conducive to allowing the expression of the hybrid protein. The culture is spun down and the resulting cell pellet broken. The total cellular protein is analysed by being run on an SDS polyacrylamide gel. The fusion proteins are identified at a position on the gel which contains no other protein. (Figure 2) Western blot analyses are also carried out on the clones which screened positive. Such analyses are carried out using serum from AIDS patients, with the result that it is possible to identify those clones expressing HTLV-III env-B-galactosidase fusion proteins (antigens) that cross-react with the HTLV-III specific antibody.

In another embodiment of this invention, lambda $_{10}$ clones harboring HTLV-III DNA are cloned

from the replicated form of the virus. As the retrovirus is replicating, double stranded DNA is being produced. Cuts are made in the cloned HTLV-III DNA with the restriction enzyme SstI. (Figure 1a) Because there are two SstI recognition sites within the LTR of HTLV-III DNA, one LTR region is not present in the cloned DNA sequence removed from the $lambda_{10}$ vector. As a result, a small (approximately 200 bp) fragment of the HTLV-III DNA is missing.

The resulting DNA is linearized and fragments are produced by digesting the linearized genomic DNA spanning the env gene region with restriction enzymes. For example, fragments are produced using Kpn or EcoRI plus HindIII, as shown in Figure 1b. The resulting 2.3kb KpnI-KpnI fragments; 1.0kbEcoRI-EcoRI fragments and 2.4Kb EcoRI-HindIII fragments are isolated by gel electrophoresis and electro-elution. These fragments are randomly sheared to produce fragments. The fragments thus produced are purified from agarose gel and DNA fragments between about 200-500 bp are eluted.

The eluted 200-500bp DNA fragments are end filled through the use of E. coli T_4 polymerase and blunt end ligated into an open reading frame expression (ORF) vector, such as pMR100. This ligation may occur at the SmaI site of the pMR100 vector, which contains two promoter regions, hybrid coding sequences of lambdaCI gene and lacI-LacZ gene fusion sequence. In the vector, these are out of frame sequences; as a result, the vector is nonproductive. The HTLV-III DNA is inserted into the vector; the correct DNA fragments will correct the reading

frame, with the result that CI-HTLV-III-B-galactosidase fusion proteins are produced. The expression of the hybrid is under the control of the lac promoter.

Based on the sequence of pMR100, it appears that if a DNA fragment insert cloned into the SmaI site is to generate a proper open reading frame between the lambdaCI gene fragment and the lac-7 fragment, the inserted DNA must not contain any stop codons in the reading frame set by the frame of the lambdaCI gene.

The hybrid molecules are then introduced into E. coli. The bacteria are plated on MacConkey agar plates to verify the phenotype of the clone. If functional B-galactosidase is being produced, the colony will appear red. The colonies are also screened with HTLV-III DNA probes, for the purpose of identifying those clones containing the insert. Clones which are positive when screened with the DNA probe and positive on the MacConkey agar plates are isolated.

The cells from the selected colonies are grown in culture. The culture is spun down and the cell pellet broken. Total cellular protein is analysed by being run on an SDS polyacrylamide gel. The fusion proteins are identified at a position on the gel which contains no other protein. (Figure 4)

Western blot analyses are also carried out on the clones which screened positive. Sera from AIDS patients are used, thus making it possible to identify those clones which express the HTLV-III-env-B-galactosidase fusion proteins (antigens) that cross-react with the HTLV-III specific antibody.

1000 clones were screened by this method; 6 were positive.

Because of the nature of the pMR100 cloning vehicle, a productive DNA insert should also be expressed as a part of a larger fusion polypeptide. HTLV-III env gene containing recombinant clones was identified by colony hybridization. The production of larger fusion polypeptides bearing functional B-galactosidase activity was verified by phenotype identification on MacConkey agar plates; by B-galactosidase enzyamatic assays and by analysis on 7.5% SDS-polyacrylamide gels. Immunoreactivity of the larger protein with antibody to HTLV-III was assessed by western blot analysis using serum from AIDS patients. These large fusion proteins also reacted with anti-B-galactosidase and anti-CI antiserum. This finding is consistent with the hypothesis that they are proteins of CI-HTLV-III-lacIZ.

The open reading frame insert fragment of HTLV-III is further analyzed by DNA sequencing analysis. Because one of the two BamHI sites flanking the SmaI cloning site in pMR100 is destroyed in the cloning step, positive clones are digested with restriction enzymes HindIII and claI to liberate the inserted HTLV-III DNA fragment. The HTLV-III ORF inserts are isolated from the fusion recombinant and cloned into M13 sequencing cloning vector mp18 and mp19 digested with HindIII and AccI. DNA sequences of the positive ORF clones are then determined.

In another embodiment of this invention, fragments of HTLV-III DNA of approximately 200-500

bps are isolated from agarose gel, end repaired with T_4 polymerase and ligated to EcoRI linker. The EcoRI linker ligated DNA is then treated with EcoRI purified from 1% agarose gel and cloned in an expression vector, gt11. This vector contains lac Z gene coding sequences into which the foreign DNA can be inserted for the generation of B-galactosidase fusion protein. The expression of the hybrid gene is under the control of lac repressor. The lac repressor gene, lac I, is carried on a separate plasmid pMC9 in the host cell, E. coli Y1090. AIDS patient serum was used to probe the gt11 library of HTLV-III genome DNA containing 1.5×10^4 recombinant phage. In a screen of 5000 recombinants, 100 independent clones that produced strong signals were isolated. The positive recombinant DNA clones were further characterized for their specific gene expression. Rabbit hyperimmune serum against P24 was also used to identify the gag gene specific clones. Nick-translated DNA probes of specific HTLV-III gene, specifically the gag gene, env gene and Px gene were used to group the positive immunoreactive clones into specific gene region.

Recombinant clones that produced strong signals with AIDS serum and contain insert DNA spanning the HTLV-III env gene region were examined in detail by mapping their insert with restriction enzymes and DNA sequencing analysis.

Another embodiment of this invention relates to the formation of RNA and RNA probes specific to the HTLV-III DNA of this invention. DNA sequences which are an entire gene or segment of a gene from HTLV-III are inserted into a vector, such as a T7 vector.

In this embodiment, the vector has the Tceu promoter from the T cell gene 10 promoter and eleven amino acids from the T cell gene 10 protein.

The vectors are then used to transform cells, such as E. coli. The T7 vector makes use of the T7 polymerase, which catalyzes RNA formation and recognizes only T7 promoter, which is the site where RNA polymerase binds for the initiation of transcription. This vector does not, however, recognize E. coli promoter. As a result, if HTLV-III DNA sequences are inserted after the promoter and polymerase genes of the T7 vector, which recognizes them to the exclusion of other signals, and a terminator is placed immediately after the HTLV-III DNA sequences, the T7 vector will direct manufacture RNA complementary to the HTLV-III DNA insert.

Monoclonal antibodies reactive with HTLV-III envelope polypeptide are produced by antibody-producing cell lines. The antibody-producing cell lines may be hybridoma cell lines commonly known as hybridomas. The hybrid cellsa re formed from the fusion of cells which produce antibody to HTLV-III envelope polypeptide and an immortalizing cell line, that is, a cell line which imparts long term tissue culture stability on the hybrid cell. In the formation of the hybrid cell lines, the first fusion partner - the antibody-producing cell - may be a spleen cell of an animal immunized against HTLV-III envelope polypeptide. Alternatively, the antibody-producing cell may be an anti-HTLV-III envelope polypeptide lymphocyte obtained from the spleen, peripheral blood, lymph nodes or other tissue. The

second fusion partner - the immortal cell - may be a lymphoblastoid cell or a plasmacytoma cell such as a myeloma cell, itself an antibody-producing cell but also malignant.

Murine hybridomas which produce monoclonal antibodies against HTLV-III envelope polypeptide are formed by the fusion of mouse myeloma cells and spleen cells from mice immunized against the polypeptide. To immunize the mice, a variety of different immunization protocols may be followed. For instance mice may receive primary and boosting immunizations of the purified polypeptide. The fusions are accomplished by standard procedures. Kohler and Milstein, (1975) Nature (London) 256, 495-497; Kennet, R., (1980) in Monoclonal Antibodies (Kennet et al., Eds. pp. 365-367, Plenum Press, NY).

The hybridomas are then screened for production of antibody reactive with envelope polypeptide.

Another way of forming the antibody-producing cell line is by transformation of antibody-producing cells. For example, a B lymphocyte obtained from an animal immunized against HTLV-III envelope polypeptide may be infected and transformeed with a virus such as the Epstein-Barr virus in the case of human B lymphocytes to give an immortal antibody-producing cell. See, e.g., Kozbor and Rodor (1983) Immunology Today 4(3), 72-79. Alternatively, the B lymphocyte may be transformed by a transforming gene or transforming gene product.

The monoclonal antibodies against HTLV-III envelope polypeptide are produced in large quantities by injecting antibody-producing hybridomas into the peritoneal cavity of mice and, after an

appropriate time, harvesting the ascites fluid which contains very high titer of homogenous antibody and isolating the monoclonal antibodies therefrom. Xenogeneic hybridomas should be injected into irradiated or athymic nude mice. Alternatively, the antibodies may be produced by culturing cells which produce HTLV-III envelope polypeptide in vitro and isolating secreted monoclonal antibodies from the cell culture medium.

This invention will now be further illustrated by the following examples. They are not intended to be limiting in any way.

EXAMPLE 1
PREPARATION OF SONICATED DNA FRAGMENTS

10 ug of gel purified HTLV-III restriction fragments were sonicated to fragment size on average of 500 bps. After sonication, the DNA was passed through a DEAE-cellulose column in 0.1XTBE in order to reduce the volume. The DEAE-bound DNA was washed with 5 ml of 0.2 M NaCl-TE (2 M NaCl, 10 mm Tris HCl pH 7.5, 1 mM EDTA) and then eluted with 1 M NaCl-TE, and ethanol precipitated. The size range of the sonicated DNA was then determined on 1.2% agarose gel. DNA fragments of desired length (200-500 bps) was eluted from the gel. T4 DNA polymerase was used to fill in and/or trim the single strand DNA termini generated by the sonication procedure. DNA fragments were incubated with T4 polymerase in the absence of added nucleotides for five minutes at 37°C to remove nucleotides from 3' end and then all 4 nucleotide precursors were added to a final

concentration of 100 uM and the reaction mixture was incubated another 30 minutes to repair the 5'-end single stranded overhang. The reaction was stopped by heat inactivation of the enzyme at 68°C for 10 minutes. DNA was phenol extracted once, ethanol precipitated and resuspended in TE.

EXAMPLE 2

CLONING OF RANDOM SHEARED DNA FRAGMENTS

The sonicated blunt end repaired HTLV-III DNA fragments were ligated into the SmaI site of the ORF expression vector pMR100 and transformed into host cell LG90 using standard transformation procedures. B-galactosidase positive phenotype of the transformant were identified by plating the transformed cell on ampicillin (25 ug/ml) containing McConkey agar plates and scoring the phenotype after 20 hours at 37°C.

EXAMPLE 3

HYBRID PROTEIN ANALYSIS

Ten milliliter samples of cells from an overnight saturated culture grown in L broth containing ampicillin (25 ug/ml) were centrifuged, the cell pellet was resuspended in 500 ul of 1.2 fold concentrated Laemmli sample buffer. The cells were resuspended by vortexing and boiling for 3 minutes at 100°C. The lysate was then repeated by being forced through a 22 guage needle to reduce the lysate viscosity. Approximately 10 ul of the protein samples were electrophoresed in 7.5% SDS-PAGE (SDS-polyacrylamide) gels.

Electrophoretic transfer of proteins from SDS-PAGE gels to nitrocellulose paper was carried out according to Towbin et. al.. After the transfer, the filter was incubated at 37°C for two hours in a solution of 5% (w/v) nonfat milk in PBS containing 0.1% antifoam A and 0.0001% merthiolate to saturate all available protein binding sites. Reactions with AIDS antisera were carried out in the same milk buffer containing 1% AIDS patient antisera that had been preabsorbed with E. coli lysate. Reactions were performed in a sealed plastic bag at 4°C for 18-24 hours on a rotatory shaker. Following this incubation, the filter was washed three times for 20 minutes each at room temperature in a solution containing 0.5% deoxycholic, 0.1 M NaCl, 0.5% triton X-100, 10 mm phosphate buffer pH 7.5 and 0.1 mM PMSF.

To visualize antigen-antibody interactions, the nitrocellulose was then incubated with the second goat antihuman antibody that had been iodinated with ^{125}I. The reaction with the iodinated antibody was carried out at room temperature for 30 minutes in the same milk buffer as was used for the first antibody. The nitrocellulose was then washed as previously described and exposed at -70°C using Kodak XAR5 film with an intensifying screen.

EXAMPLE 4

SCREENING OF THE HTLV-III ORF LIBRARY BY COLONY HYBRIDIZATION

E. coli LG90 transformants were screened with HTLV-III DNA probes containing the DNA regions of interest (e.g. HTLV-III gag, env or Px gene specific

sequences). Colonies were grown on nitrocellulose filter and screened according to the procedure of Grunstein and Hogness by using a nick-translated HTLV-III DNA as hybridization probe.

The DNA fragment was in general exercise by restriction endonuclease digestion, gel purified, and ^{32}P-labeled to a specific activity of 0.5×10^8 cpm/ug by nick-translation (Rigby, P.W.J. et al., J. Mol. Biol. 113, 237 (1977). Duplicate nitrocellulose filters with DNA fixed to them were prehybridized with 6xSSC (0.9 M NaCl/0.09 M sodium citrate, pH 7.0), 5X Denhardt's solution (Denhardt's solution: 0.02% each of polyvinylpyrrolidone, Ficoll and bovine serum albumin) 10 ug of denatured sonicated E. coli DNA per ml at 55°C for 3-5 hours. The filters were then placed in a fresh sample of the same solution to which the denatured hybridization probe had been added. Hybridization was permitted to take place at 68°C for 16 hours. The filters were washed repeatedly in 0.3XSSC at 55°C, and then exposed to x-ray film.

Industrial Applicability

This invention has industrial applicability in screening for the presence of HTLV-III DNA in body fluids and the diagnosis of AIDS.

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substantces and procedures described herein. Such equivalents are considered to be within the

scope of this invention and are covered by the following claims.

CLAIMS

1. Immunoreactive HTLV-III polypeptide expressed by cells transformed with a recombinant vector containing HTLV-III cDNA.

2. A polypeptide of Claim 1 wherein said HTLV-III cDNA encodes an env gene sequence.

3. A polypeptide of Claim 2 wherein which is immunoreactive with sera of patients with acquired immunodeficiency syndrome.

4. Isolated HTLV-III envelope polypeptide.

5. Isolated cDNA encoding an HTLV-III gene.

6. cDNA of Claim 5 encoding the HTLV-III env gene.

7. Isolated cDNA encoding for an HTLV-III polypeptide which is immunoreactive.

8. Isolated cDNA of Claim 7 coding for an envelope polypeptide which is immunoreactive.

9. A DNA probe comprising a DNA sequence coding a portion of the HTLV-III genome.

10. A DNA probe of Claim 9 wherein the DNA sequence encodes at least a portion of the env gene.

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11. A hybrid protein comprising an HTLV-III polypeptide linked to at least one other polypeptide.

12. A hybrid protein of Claim 11 comprising an HTLV-III polypeptide linked to an indicator polypeptide.

13. A hybrid protein of Claim 12 wherein said indicator polypeptide comprises beta-galactosidase.

14. An isolated RNA transcript of the env gene of HTLV-III.

15. An isolated RNA transcript of Claim 14 having a label which emits a detectable signal.

16. An isolated RNA transcript of Claim 15 wherein said label comprises a radioisotope.

17. A recombinant vector containing HTLV-III DNA capable of expression upon insertion into host cells.

18. OmpA vector containing HTLV-III cDNA.

19. pMR 100 vector containing HTLV-III cDNA.

20. A method of producing HTLV-III polypeptide, comprising the steps of:

    a. cleaving HTLV-III cDNA to produce DNA fragments;

b. inserting the DNA fragments into an expression vector to form a recombinant vector;

c. transforming an appropriate host cell with the recombinant vector; and

d. culturing the transformed host cell under conditions sufficient for expression of the polypeptide coded for by the inserted HTLV-III DNA.

21. A method of Claim 20 wherein the cleaving step comprises digesting the HTLV-III cDNA with restriction endonucleases to produce restriction fragments of cDNA.

22. A method of Claim 20 wherein the cleaving step comprises shearing the HTLV-III cDNA to produce cDNA fragments.

23. A method of producing HTLV-III envelope polypeptide, comprising the steps of:

a. cleaving HTLV-III genomic cDNA with the restriction endonuclease SstI;

b. digesting the cleaved cDNA with restriction endonucleases sufficient to generate restriction fragments which encompass at least a portion of the env gene;

c. isolating the restriction fragments;

d. producing DNA fragments of about 200-500 base pairs in length from the restriction fragments;

e. isolating the DNA fragments of about 200-500 base pairs;

f. inserting the isolated fragments into the open reading frame expression vector pMR100 for production of hybrid proteins comprising an env gene product and beta-galactosidase;

g. transforming lac z^- E. coli cells with the vector;

h. plating the transformed cells on MacConkey agar plates, maintaining the plates under conditions sufficient for the formation of colonies and selecting cell colonies exhibiting a red color;

i. culturing transformed cells from the selected colonies under conditions which allow expression of the hybrid protein;

j. obtaining cellular protein from the cultured transformed cells;

k. separating the cellular protein obtained;

l. contacting the separated protein with sera from AIDS patients to identify protein which is immunoreactive with the sera; and

m. isolating the immunoreactive protein.

/

24. A method of Claim 23, further comprising the step of separating the env gene expression product from the remainder of the hybrid protein.

25. A fusion protein produced by the method of Claim 23./

26. A HTLV-III envelope polypeptide produced by the method of Claim 24.

27. Antibody specifically reactive with HTLV-III envelope polypeptide.

28. An antibody of Claim 27 which is monoclonal.

29. Anibody specifically reactive with HTLV-III polypeptide produced by recombinant DNA techniques.

30. An antibody of Claim 29 which is monoclonal.

31. An immunoassay for the detection of HTLV-III employing antibody which reacts specifically with HTLV-III polypeptide produced by recombinant DNA techniques.

32. An immunoassay for the detection of HTLV-III employing antibody which reacts specifically with HTLV-III envelope polypeptide.

33. An immunoassay of Claim 32 wherein said antibody is monoclonal.

34. A method for detecting the presence of HTLV-III in a bodily fluid comprising the steps of:

a. contacting an immunoadsorbent comprising a solid phase having an antibody which specifically binds HTLV-III polypeptide with the bodily fluid;

b. separating the immunoadsorbent and the fluid;

c. contacting the immunoadsorbent with a labeled antibody which specifically binds HTLV-III polypeptide; and

d. measuring the amount of label associated with the immunoadsorbent to determine the presence of HTLV-III.

35. An assay kit comprising an antibody which reacts specifically iwth HTLV-III polypeptide bound to a solid phase and a labeled antibody which reacts specifically HTLV-III polypeptide.

36. A method of determining the presence of antibodies against HTLV-III in a bodily fluid comprising the steps of:

a. contacting an immunoadsorbent comprising an HTLV-III polypeptide bound to a solid phase with a bodily fluid;

b. separating the immunoadsorbent from the bodily fluid;

c. contacting the immunoadsorbent with a labeled HTLV-III polypeptide; and

d. determining the amount of labeled polypeptide bound to immunoadsorbent as an indication of antibody to HTLV-III.

37. A kit for determining the presence of antibody against HTLV-III in a bodily fluid comprising:

a. an immunoadsorbent comprising a HTLV-III polypeptide bound to a solid phase; and

b. labeled HLTV-III polypeptide.

38. A method of detecting HTLV-III nucleic acid in a bodily fluid comprising the steps of:

a. adsorbing the nucleic acid in a bodily fluid onto an adsorbent;

b. denaturing the adsorbed nucleic acid;

c. contacting the adsorbed nucleic acid with a HTLV-III DNA or RNA probe; and

d. determining if the probe hybridizes with the adsorbed nucleic acid.

39. A method of Claim 38 wherein the bodily fluid is a cell lysate.

40. A hybridoma cell line which produces antibody specifically reactive with HTLV-III envelope polypeptide.

CLONING AND EXPRESSION OF HTLV-III DNA

Abstract

The production of immunoreactive polypeptides from HTLV-III by recombinant DNA methods is disclosed. Such polypeptides can be employed in immunoassays to detect HTLV-III.

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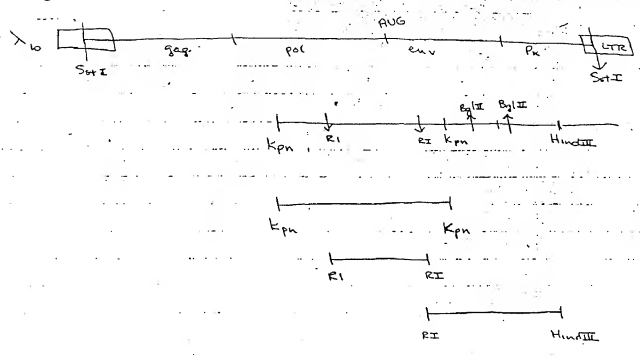
FIGURE 1



1b

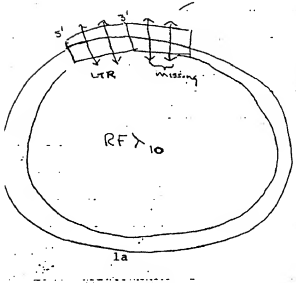


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FIGURE 2

FIGURE 3 (A)

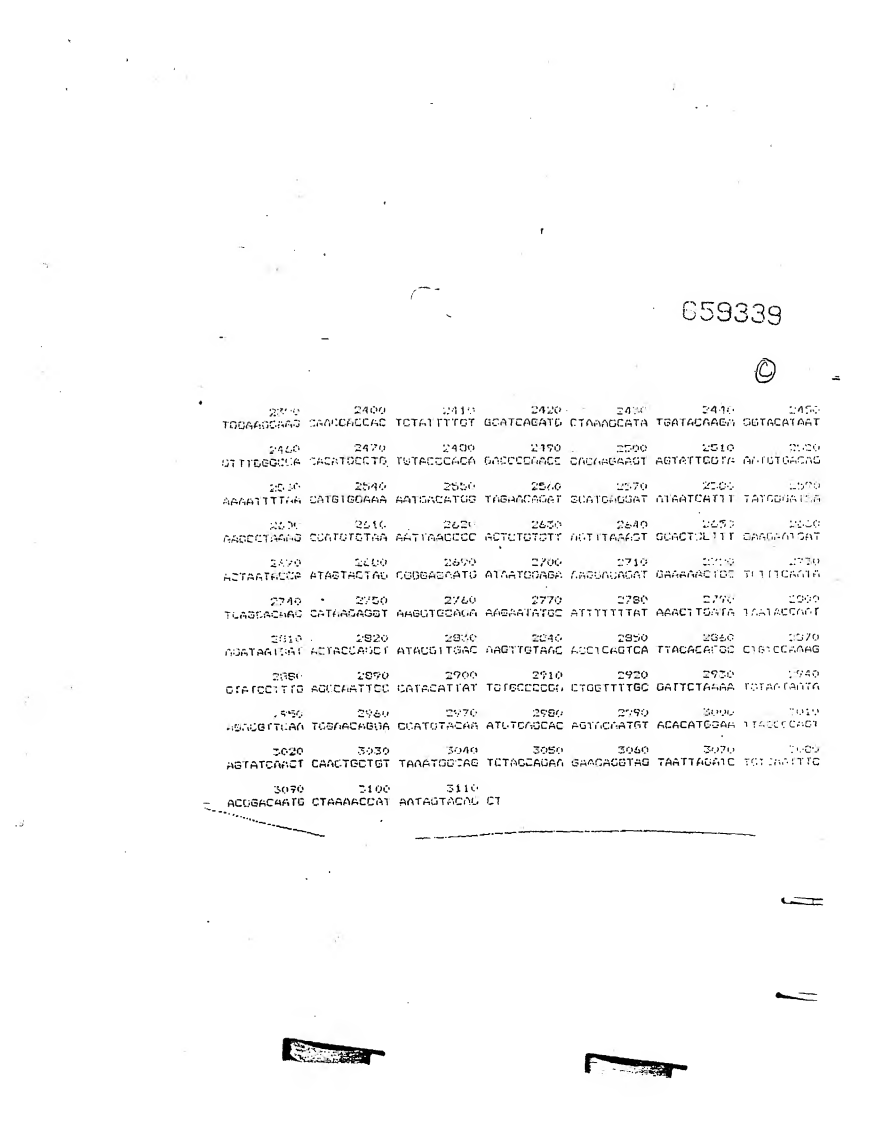

FIGURE 4

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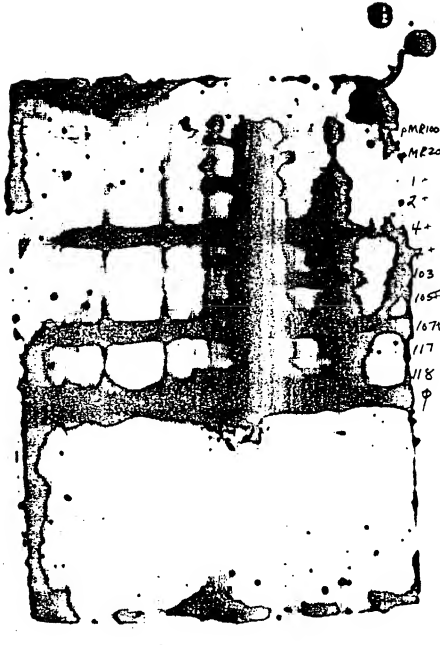
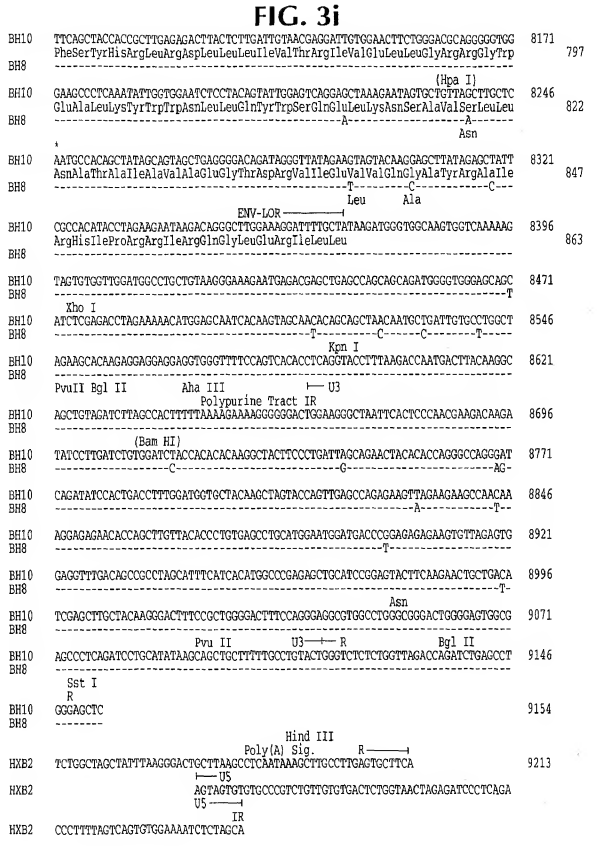


FIG. 3a


CLONE
NUCLEOTIDE POSITION
AMINO ACID RESIDUE
U3
IR
BH10
BH8
(Bam HI)
TATA BOX
Pvu II
U3
R
Bgl II
Sgt I
HXB2
Hind III
U5
tRNA-lysine
Leader sequence
GAG p17
Hind III
BH5

FIG. 3i


BH10 TTCAGCTACCACCGCTTGAGAGACTTACTCTTGATTGTAACGAGGATTGTGGAACTTCTGGGACGCAGGGGGTGG 8171
PheSerTyrHisArgLeuArgAspLeuLeuLeuIleValThrArgIleValGluLeuLeuGlyArgArgGlyTrp 797
BH8
(Hpa I)
BH10 GAAGCCCTCAAATATTGGTGGAATCTCCTACAGTATTGGAGTCAGGAGCTAAAGAATAGTGCTGTTAGCTTGCTC 8246
GluAlaLeuLysTyrTrpTrpAsnLeuLeuGlnTyrTrpSerGlnGluLeuLysAsnSerAlaValSerLeuLeu 822
BH8
Asn
BH10 AATGCCACAGCTATAGCAGTAGCTGAGGGGACAGATAGGGTTATAGAAGTAGTACAAGGAGCTTATAGAGCTATT 8321
AsnAlaThrAlaIleAlaValAlaGluGlyThrAspArgValIleGluValValGlnGlyAlaTyrArgAlaIle 847
BH8
Leu Ala
ENV-LOR
BH10 CGCCACATACCTAGAAGAATAAGACAGGGCTTGGAAAGGATTTTGCTATAAGATGGGTGGCAAGTGGTCAAAAAG 8396
ArgHisIleProArgArgIleArgGlnGlyLeuGluArgIleLeuLeu 863
BH8
BH10 TAGTGTGGTTGGATGGCCTGCTGTAAGGGAAAGAATGAGACGAGCTGAGCCAGCAGCAGATGGGGTGGGAGCAGC 8471
BH8
Xho I
BH10 ATCTCGAGACCTAGAAAAACATGGAGCAATCACAAGTAGCAACACAGCAGCTAACAATGCTGATTGTGCCTGGCT 8546
BH8
Kpn I
BH10 AGAAGCACAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTACAAGGC 8621
BH8
PvuI Bgl II Aha III U3
Polypurine Tract IR
BH10 AGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTAATTCACTCCCAACGAAGACAAGA 8696
BH8
(Bam HI)
BH10 TATCCTTGATCTGTGGATCTACCACACACAAGGCTACTTCCCTGATTAGCAGAACTACACACCAGGGCCAGGGAT 8771
BH8
BH10 CAGATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGAGAAGTTAGAAGAAGCCAACAA 8846
BH8
BH10 AGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGATGACCCGGAGAGAGAAGTGTTAGAGTG 8921
BH8
BH10 GAGGTTTGACAGCCGCCTAGCATTTCATCACATGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCTGACA 8996
BH8
Asn
BH10 TCGAGCTTGCTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGCG 9071
BH8
Pvu II U3 R Bgl II
BH10 AGCCCTCAGATCCTGCATATAAGCAGCTGCTTTTGCCTGTACTGGGTCTCTCTGGTTAGACCAGATCTGAGCCT 9146
BH8
Sst I
R
BH10 GGGAGCTC 9154
BH8
Hind III
Poly(A) Sig. R
HXB2 TCTGGCTAGCTATTTAAGGGACTGCTTAAGCCTCAATAAAGCTTGCCTTGAGTGCTTCA 9213
U5
HXB2 AGTAGTGTGTGCCCGTCTGTTGTGTGACTCTGGTAACTAGAGATCCCTCAGA
U5
IR
HXB2 CCCTTTTAGTCAGTGTGGAAAATCTCTAGCA